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R E M A R K S

Claims 1, 2, 4, 5 and 7-20 are pending. No new matter has been added by way of the present submission.

For instance, the specification has been amended to correct inadvertent typographical and grammatical errors. The specification has also been amended to reference that the present application claims priority of Application No. 2000-374145, filed in Japan on December 8, 2000 under 35 U.S.C. §119.

Concerning amendments made to the claims, claims 1, 2 and 10 were amended to replace the recitation of "react" with "bind." Also, in claims 8, 9, 10 and 16, the recitation of "according to" has been replaced with "defined in" as a matter of preference. Claim 1 was also amended to include textual subject matter taken from originally filed claim 10. Claim 4 was amended to replace "and immunogen" with "an immunogen" and to replace "having" with "comprising". Claim 8 was amended to replace "measuring" with "detecting or quantifying" as supported by the present specification at page 9, lines 22-23. Claim 9 was amended to be of properly dependent format. Claim 10 was amended to clarify the language concerning the nature of the abnormal type prion. Claim 11 was amended to clarify that the peptide is immobilized on a carrier. Claims 12 and 14 were amended to depend upon claim 10.

Claim 12 and claim 15 were amended to clarify the nature of the immunogen. New claim 18 is supported by originally filed claims 1 and 13 as well as the present specification at page 2, lines 22-25, and page 8, lines 6-10. New claim 19 is the same as pending claim 13, with the exception that the "E3 region" is also listed as supported by the present specification at page 8, line 10, and Figure 1. New claim 20 is the same as new claim 18, with the exception that the "E3 region" is also listed as supported by the present specification at page 8, line 10, and Figure 1. Accordingly, no new matter has been added.

In view of the following remarks, Applicants respectfully request that the Examiner withdraw all rejections and allow the currently pending claims.

Issue of Priority

At page 2, subheading 3 of the outstanding Office Action the Examiner has acknowledged the claim for foreign priority based upon JP 2000-374145. However, the Examiner notes that a certified English translation of the application has not been filed. Applicants submit that it is currently unnecessary to submit a certified English translation of the priority document.

The Examiner further asserts that the claim of priority of the Japanese application should be listed in the first paragraph of the specification. Applicants have amended the specification to reflect the claim of priority. However, Applicants submit that all requirements concerning priority have been met, even without the current amendment to the specification.

Accordingly, the issues raised with respect to priority are moot. Reconsideration and withdrawal thereof are requested.

Objections to the Specification

At pages 2-3 of the outstanding Office Action the Examiner has outlined several objections to the specification. Applicants traverse and submit that each of these objections have been dealt with. Below, Applicants individually address each of these objections.

The Examiner has objected to the recitation of "almost localized in nerve system, and progressively aggravate to death." Applicants have replace this phrase with "mostly localized in the nerve system, and progressively worsened and lead to death."

The Examiner has pointed out that at page 2, lines 17 and 19 and page 9, line 6 the recitation of "antigenecity" should be "antigenicity". Applicants have corrected this issue.

At page 2, line 20 of the specification, the Examiner queries "what is meant by 'demanded.'" Applicants have addressed this issue by deleting the recitation of ", is demanded" at page 2, lines 19-20 and inserting the phrase "there is a demand for the" after the word "thus," at page 2, line 17.

The Examiner has objected to the recitation of "Because of these" at page 3, line 10. Applicants have rephrased this to recite "Because of the above".

The Examiner has objected to the recitation of "regions are discontinuous each other in" at page 4, line 11. Applicants have replaced this phrase with "regions are discontinuous with each other in the".

The Examiner has asserted that at page 5, line 6, the recitation of "reacts" should be replaced with "react". Applicants have adopted this suggested amendment.

The Examiner has asserted that at page 6, line 11, the recitation of "means fragment" should be replaced with "means a fragment". Applicants have adopted this suggested amendment.

The Examiner has asserted that it is unclear what is meant by "the present inventors thought a structure" at page 7, line 12. Applicants have replaced the recitation of "thought" with "theorized".

The Examiner has asserted that at page 7, lines 17, 18 and 21, it is not understood what is meant by "helix 2" and "helix 3". Applicants traverse and submit that the terms "helix 2" and "helix 3" are well known in the art. To emphasize this point, Applicants have attached a reference, NATURE VOL 382, 11, JULY 1996, pages 180-182. As shown in Fig. 3 on page 182, helix 2 (H2) is a.a. 179-193 and helix 3 (H3) is a.a. 200-217 of the prion protein.

The Examiner has also asserted that it is unknown what is meant by the recitation of "E3 region" at page 8, line 10. Again, Applicants submit that this is a term well known in the art. In fact, reference to Figure 1 of the present application reveals a reference to "E3." Moreover, as expressly stated on page 7, lines 23-24 of the specification, E1, E2 and E3 constitute the epitope of 15B3. Further, page 11, lines 16-19 of the specification states

> Korth et al. analyzed the epitope of the bovine prion protein by using a recombinant antigen expressed by a genetic recombination technique, and reported that the major antigenic regions of the prion protein are the above-mentioned three regions, i.e., E1, E2 and E3 regions (Korth et al., *supra*).

Thus, E3 region is one of the three epitopes of 15B3. The 15B3 epitope is well-known. To further emphasize this point, Applicants have attached a reference, Korth et al., NATURE VOL 390, 6, NOVEMBER 1997, pages 74-77 cited in the present specification on page 6, line 23, and on page 11, line 19. The 15B3 epitope is shown in Fig. 2 and on page 75, right column, line 27 to page 76, right column, line 1. E3 is designated "15B3-3" in Fig. 2b of the reference, which shows the amino acid sequence thereof. Since the E1 and E2 regions are specifically described in the present specification, and since the three epitopes of 15B3 are well-known as shown in Korth et al., cited in the present specification, those skilled in the art readily understand what the E3 region is as well as its amino acid sequence. Please note, however, that Applicants for shortness sake created the designations "E1", "E2" and "E3". In Korth et al., they are designated "15B3-1", "15B3-2" and "15B3-3", respectively.

The Examiner has also asserted that at page 9, line 15, the recitation of "does" should be replaced with "do". Applicants have adopted this suggested amendment.

The Examiner points to page 13, line 22 and states that if "ABC reagent" is a registered trademark, it should be so noted. "ABC reagent", it is a trademark of Vector Laboratories, Inc. at Burlingame, CA, USA. Applicants have placed the "®" designation as well as the address, in the specification.

The Examiner asserts that at page 20, line 9, the recitation of "regions are discontinuous each other in" is unclear. Applicants have replaced this phrase with "regions are discontinuous with each other in the".

In view of the above, Applicants submit that each of the Examiner's objections to the specification are moot. Reconsideration and withdrawal thereof are requested.

Objections to the Claims

The Examiner has objected to the claims for the reasons recited at page 3 of the outstanding Office Action. Applicants respectfully traverse.

The Examiner has objected to claim 4 asserting that in line 3, "and" should be "an". Applicants have adopted this suggested amendment.

The Examiner has objected to claim 10 asserting that in line 5 the recitation of "discontinuous each other in the primary" should be "discontinuous with each other in the primary". Additionally, at line 7 of claim 10 the Examiner has suggested inserting the word "to" after "ligated". Applicants have adopted these suggested amendments.

In view of the above, Applicants submit that each of the Examiner's objections to the claims are moot. Reconsideration and withdrawal thereof are requested.

Issues Under 35 U.S.C. §112, second paragraph

The Examiner has rejected various claims under 35 U.S.C. § 112, second paragraph for the reasons recited at pages 4-6 of the outstanding Office Action. Applicants respectfully traverse each of these rejections.

First, the Examiner has rejected claims 6 and 9 asserting that it is unclear what new criticality is added by the language of dependent claim 6. Claim 6 has been cancelled, thus, this rejection is moot.

Second, the Examiner has rejected claim 8 and suggested replacing the recitation of "measuring" with "identifying and quantifying". However, Applicants submit that the suggested amendment is not proper for the reasons explained below:

With regard to claim 8, Applicants submit that the phrase "identifying and quantifying" is not completely suitable because detection is arguably excluded from the scope of the claim. On page 9, lines 22-23, it is expressly stated that the term

"measure" includes both detection and quantification. However, "identifying" is not mentioned in the specification and nothing on the record indicates that it has the same meaning as "detecting." This sentence in the specification is intended to mean that detection and quantification are included in the term "measure." Therefore, if mere detection and not quantification is intended to be included in the scope of claim 8, the suggested language fails. That is, if the phrase "identifying and quantifying" (emphasis added) is used, it would seem that mere detection (not quantification) may arguably be excluded from the scope of claim 8. Please note that in the actual working examples described in the specification, detection alone was carried out. Accordingly, Applicants have amended the phrase in claim 8 to recite "detecting or quantifying".

Third, the Examiner has rejected claim 11 asserting that the claim appears to be improperly redefining the meaning of the immunogen of claim 10. Applicants have addressed this issue by way of the present amendment.

Fourth, the Examiner has rejected claim 12 for similar reasons to the reasons for rejecting claim 11. Applicants have amended claim 12 to address this issue.

Fifth, the Examiner further asserts that the recitation of "plurality of kinds" of said peptide in claim 12 is indefinite since the scope of "plurality of kinds" is not defined in the specification. Applicants submit that claim 12 has been amended to recite "at least two types" of said peptide.

Sixth, the Examiner has rejected claim 14 asserting that the recitation of "said peptide has an amino acid sequence shown in SEQ ID NO: 1" should be amended to recite "said peptide comprises the amino acid sequence shown in SEQ ID NO: 1". Applicants have adopted this suggested amendment. Applicants have also made similar changes to claims 4 and 15.

Seventh, the Examiner has rejected claims 1-16 for the recitation of a monoclonal antibody, which "reacts" with abnormal type prion but does not substantially "react" with normal type prion. The Examiner asserts that the terminology "reacts" is indefinite and as suggested to replacing the word with "binds." Applicants have adopted this suggested amendment.

Eighth, the Examiner has rejected claim 3 asserting that the recitation of "pretreatment" is unclear. Claim 3 has been cancelled, thus, this rejection is moot.

In view of the above, Applicants submit that each of the Examiner's rejections under 35 U.S.C. § 112, second paragraph are moot. Reconsideration and withdrawal thereof are requested.

Issues Under 35 U.S.C. §102(b)

The Examiner has rejected claims 1-3 and 6-8 under 35 U.S.C. §102(b) as being anticipated by Prusiner et al., USP 5,846,533 (hereinafter referred to as Prusiner '533). Applicants respectfully traverse this rejection.

Prusiner '533 allegedly discloses a monoclonal antibody which specifically binds *in situ* with abnormal prion, but not normal prion. However, claim 1 requires that the anti-abnormal type prion monoclonal antibody originate from an animal immunized with an immunogen including a peptide consisting essentially of a plurality of regions in said abnormal type prion, which regions are discontinuous with each other in the primary amino acid sequence of said abnormal type prion, and which regions are ligated to each other in said peptide. Prusiner '533 fails to suggest or disclose such a monoclonal antibody, according, there exists no anticipation. Reconsideration and withdrawal of this rejection are respectfully requested.

Issues Under 35 U.S.C. §103(a)

The Examiner has rejected claim 9 under 35 U.S.C. §103(a) as being obvious over Prusiner '533. Applicants respectfully submit

that Applicants have distinguished independent claim 1 from the disclosure of Prusiner '533. Accordingly, the same distinction applies to the present rejection. Without a suggestion of the subject matter of claim 1, the subject matter of claim 9 remains non-obvious. Reconsideration and withdrawal of this rejection are respectfully requested.

In view of the above, Applicants respectfully submit that the present claims define subject matter which is patentable over the cited art. Reconsideration and withdrawal of all outstanding objections and rejections is respectfully requested.

If the Examiner has any questions or comments, please contact Craig A. McRobbie, Registration No. 42,874 at the offices of Birch, Stewart, Kolasch & Birch, LLP.

Pursuant to 37 C.F.R. §§ 1.17 and 1.136(a), Applicants respectfully petition for a three (3) month extension of time for filing a reply in connection with the present application, and the required fee of $950.00 is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

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Attachments: Copy of Nature, Volume 382, 11 July 1996, pp. 180-182; and
Copy of Nature, Volume 390, 6 Nov. 1997, pp. 74-77

surface of the antennal lobes through a small window cut in the head cuticle; these experiments were done without the experimenter knowing whether the drop contained PCT or saline. In the second method (group 2, Table 1), 0.1 nl saline or picrotoxin (100 μM–1 mM in saline) was injected directly into the antennal lobes through a small window in the head just above the base of each antenna using a Picospritzer (General Valve)[26]. Injections gave the same results as topical applications, although PE response rates were reduced, as commonly observed after extensive surgery. After a time t_1 (10, 45, 60 or 90 min) for recovery, animals were trained by using the following protocol[13,14]: 6 paired presentations of odorant (4-s pulse into a vented air stream) and sucrose (0.4 μl of 1.25 M solution for group 1, 2 μl of 2 M solution for group 2, presented to the antenna and the proboscis 3 s after odorant pulse onset), every 2 min (group 1) or 30 s (group 2). Animals showing a PE response in each trial were selected to receive 2 or 3 extinction (odour only) trials (one with each of the 2 or 3 test odours; see below) 90 min (group 1) or 60 min (group 2) after conditioning. The odorants used for conditioning were 1-hexanol or 1-octanol. Groups were counterbalanced to contain roughly equal numbers of bees trained with either alcohol. The odours used for testing (1-octanol, 1-hexanol, geraniol) were presented to each animal in a randomized order. Generalization between the alcohols and geraniol is typically low[16]. We used the percentage of subjects that responded to an extinction test as the response measure. Results were compared with χ^2 statistics because behavioural data were categorical (PE or no PE). Statistical values are one-tailed because generalization responses were not expected to exceed the response levels to conditioned stimuli.

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Prion (PrP^{Sc})-specific epitope defined by a monoclonal antibody

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Prions are infectious particles causing transmissible spongiform encephalopathies (TSEs). They consist, at least in part, of an isoform (PrP^{Sc}) of the ubiquitous cellular prion protein (PrP^{C}). Conformational differences between PrP^{C} and PrP^{Sc} are evident from increased β-sheet content and protease resistance in PrP^{Sc} (refs 1–3). Here we describe a monoclonal antibody, 15B3, that can discriminate between the normal and disease-specific forms of PrP. Such an antibody has been long sought as it should be invaluable for characterizing the infectious particle as well as for diagnosis of TSEs such as bovine spongiform encephalopathy (BSE) or Creutzfeldt–Jakob disease (CJD) in humans. 15B3 specifically precipitates bovine, murine or human PrP^{Sc}, but not PrP^{C}, suggesting that it recognizes an epitope common to prions from different species. Using immobilized synthetic peptides, we mapped three polypeptide segments in PrP as the 15B3 epitope. In the NMR structure of recombinant mouse PrP, segments 2 and 3 of the 15B3 epitope are near neighbours in space, and segment 1 is located in a different part of the molecule. We discuss models for the PrP^{Sc}-specific epitope that ensure close spatial proximity of all three 15B3 segments, either by intermolecular contacts in oligomeric forms of the prion protein or by intramolecular rearrangement.

PrP-null mice were immunized with full-length recombinant bovine PrP. After fusion of spleen cells with myeloma cells, we selected ~50 hybridoma cells that produced monoclonal antibodies recognizing either native bovine PrP^{Sc} (PrP^{BSE}) immobilized on nitrocellulose or recombinant bovine PrP (rbPrP) in an enzyme-linked immunosorbent assay (ELISA). One of these antibodies (15B3) was selected for binding to protease-digested BSE brain homogenates; a second (6H4) efficiently recognized recombinant PrP. On western blots, 6H4 recognized rbPrP, as well as bovine, human, mouse and sheep PrP^{C}, whereas 15B3 did not react with any form of PrP (results not shown). To determine the reactivity of these antibodies with native PrP^{C} and PrP^{Sc}, we immunoprecipitated PrP from brain homogenates of normal and BSE-infected cattle. The precipitated proteins were then analysed on western blots using a rabbit polyclonal antiserum to rbPrP (Fig. 1). The 6H4 antibody precipitated PrP from BSE as well as from normal brain homogenates; 15B3 precipitated only PrP from brain homogenates of BSE-diagnosed cattle (Fig. 1a). Upon proteinase K treatment, normal PrP is completely digested, whereas the 33K–35K form of PrP^{Sc} is shortened to 27K–30K (PrP 27–30), probably as a result of

degradation of the amino-terminal segment of residues 23–90, analogous to hamster PrP^{Sc} (ref. 3). Digestions of brain homogenates or immunoprecipitates with proteinase K are shown in Fig. 1b. Proteinase K digestion of BSE homogenates or the immunoprecipitate with 15B3 yields the PrP^{BSE}-specific band of 27K–30K (Fig. 1b). Not all of the immunoprecipitated PrP was protease-resistant, suggesting that 15B3 recognizes multiple forms of disease-specific PrP with different sensitivities to proteinase K. Apparently, PrP with properties characteristic for PrP^{Sc} but without protease resistance occurs as an intermediate in the generation of fully proteinase-resistant PrP^{Sc} (ref. 4). No PrP 27–30 was found in normal homogenates or immunoprecipitates with protein A only (Fig. 1b) or 6H4 (not shown). 15B3 therefore seems to be a PrP^{BSE}-specific antibody, even though we immunized with recombinant bovine PrP. Injection of rbPrP into Tg20 mice overexpressing mouse PrP^{5} has not produced disease for 430 days, whereas two out of four mice injected with a homogenate from the medulla of a BSE-affected cow have come down with TSE at 388 and 426 days (A.R., C.K. and B.O., unpublished results). In addition, it has been reported that recombinant PrP is not infectious[6]. Recombinant PrP is also not protease-resistant, which is a hallmark of PrP^{Sc} (C.K., unpublished observation)[7,8]. The model of Lansbury and Caughey[9], which postulates that the two isoforms of PrP are in a dynamic equilibrium, provides a possible explanation for these findings. By immunizing with large amounts of normal PrP, a small portion of the protein might, according to this hypothesis, have been in the scrapie-specific conformation when triggering the immune response. Alternatively, recombinant PrP molecules might transiently associate (see below and Fig. 3b), and thereby form the prion-specific epitope when acting as an immunogen.

We further analysed the species specificity of 15B3 using mouse scrapie-infected brain homogenates (Fig. 1c) and brain homogenates from CJD type-1 patients (Fig. 1d)[10]. For comparison, the mouse brain homogenates of PrP-null as well as normal and scrapie-infected wild-type mice, and the immunoprecipitates corresponding to twice the amount of the homogenates are shown (Fig. 1c). Mouse PrP^{Sc} could be efficiently precipitated by 15B3, as indicated by the presence of PrP 27–30 after the digestion with proteinase K (Fig. 1c, lane a). When brain homogenate was treated with proteinase K before the precipitation, 15B3 was also able to precipitate PrP 27–30 (Fig. 1c, lane b), indicating that the N-terminal segment 23–90 is not critical for binding of 15B3 to PrP^{Sc}, even though precipitation of intact PrP^{Sc} appeared to be more efficient than that of PrP 27–30. Proteinase K digestion causes the formation of large aggregates (scrapie-associated fibrils) which may mask the 15B3 epitope. Surprisingly, 15B3 also specifically recognized PrP^{CJD} from sporadic CJD cases but not human PrP^{C} (Fig. 1d), even though the amino-acid sequence in the regions of the 15B3 epitope is not fully conserved (see below, and Fig. 2b).

The epitopes recognized by the two antibodies were determined by using a gridded array of synthetic peptides consisting of 104 13-residue peptides sequentially shifted in steps of two amino acids and covering the whole mature bovine PrP sequence. A single linear epitope (DYEDRYYRE; corresponding to positions 144–152 of human PrP[11]) was mapped for 6H4, whereas three distinct peptide sequences were found to form the 15B3 epitope (amino acids

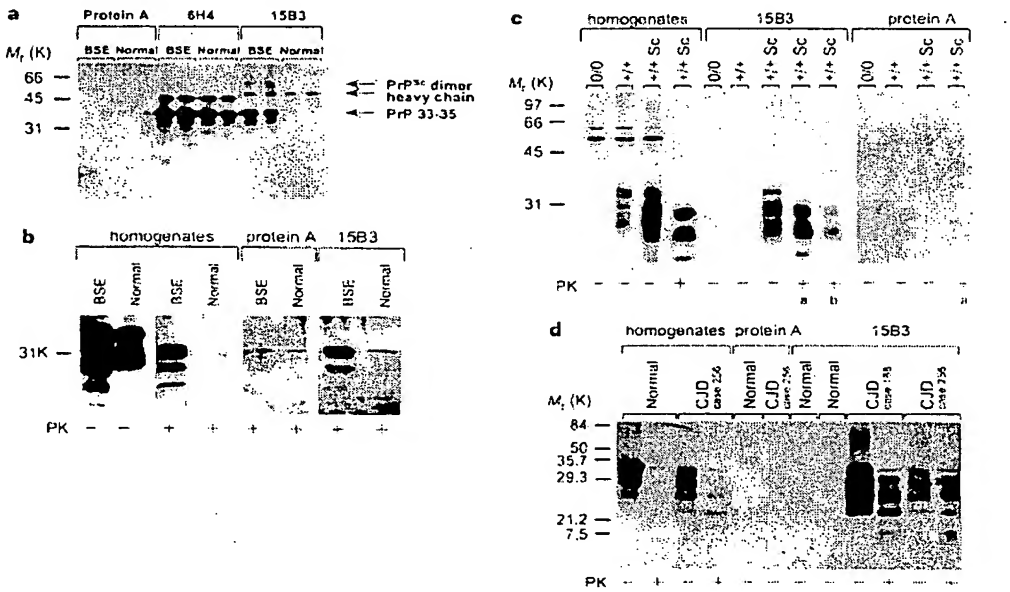

Figure 1 Immunoprecipitation of bovine, mouse and human PrP with monoclonal antibodies 15B3 and 6H4. **a**, The supernatant of a centrifuged homogenate from the medulla of two different BSE-diagnosed or two normal animals was incubated with antibodies 6H4 or 15B3. Antibodies were precipitated with protein A- (15B3) or protein G-agarose (6H4). As a control, protein A only was incubated without antibodies. Precipitates were analysed on a western blot for the presence of PrP using a polyclonal rabbit antiserum to bovine PrP and goat-anti-rabbit Ig coupled to alkaline phosphatase. Signals were developed with chemiluminescence substrates. Crossreaction of the secondary antibody with immunoprecipitated mouse immunoglobulins leads to the prominent band at about 50K. Note the 60K band characteristic for PrP^{Sc} in the 15B3 but not in the 6H4 immunoprecipitations[25]. **b**, Proteinase K digestion of PrP^{BSE} immunoprecipitated with mA 15B3. Undigested and digested bovine brain homogenates were compared to proteinase K digested immunoprecipitates with protein A-agarose only or with 15B3. The sharp band at 31K represents a crossreactivity of the secondary antibody with proteinase K. The same immunoprecipitates and method of analysis were used as in **a**. **c**, Immunoprecipitation of mouse PrP^{Sc} with mAb 15B3. Homogenates from PrP-null mice (0/0) or wild-type mice (normal (+/+) or scrapie-infected (+/+Sc)) were immunoprecipitated with mAB 15B3 or protein A-agarose only and analysed by western blotting as described. Digestion with proteinase K after (a) or before (b) the immunoprecipitation is indicated. Detection of PrP was done as described. **d**, Immunoprecipitation of human PrP^{CJD} with mAb 15B3. Brain homogenates (cerebellum) from normal persons or CJD patients type 1 (ref. 10) were immunoprecipitated and analysed as described for **a**. Two representative examples from a total of 4 normal persons and 4 CJD cases are shown.

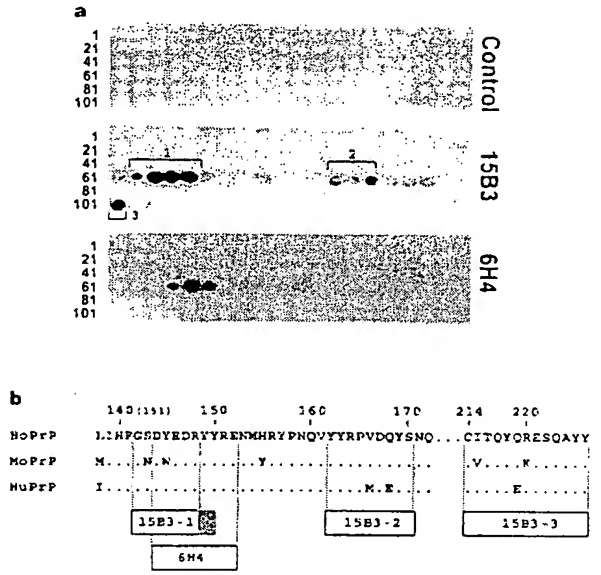

Figure 2 Determination of epitopes for mAbs 15B3 and 6H4. A gridded array of synthetic peptides corresponding to bovine PrP was incubated with 15B3, 6H4 or with secondary antibody only (peroxidase-labelled goat anti-mouse Ig; control). Bound antibody was visualized with chemiluminescence. Each spot corresponds to a 13-amino-acid peptide, which is shifted by two amino acids along the bovine PrP sequence relative to the previous peptide. Peptides were covalently attached at the C terminus to a cellulose support. A total of 104 peptides were used to cover the whole bovine PrP sequence including the six octapeptide repeat sequences[21]. **b**, Minimal sequences recognized by 15B3 or 6H4 antibodies in the array of synthetic bovine PrP peptides. The polypeptide segments of the 15B3 epitope are numbered as they occur in the amino acid sequence. The first 15B3 segment extends by two amino acids C-terminally (grey box) if spot number 62, which binds 15B3 only weakly, is excluded. The numbering of the sequence is according to human PrP[11]; the number in brackets indicates the position in the bovine PrP sequences used for the construction of the array of synthetic peptides. Differences with the human and mouse PrP sequences are indicated.

142–148, 162–170, and 214–226; Fig. 2a, b). The relative positions of these partial epitopes in the amino-acid sequence revealed an overlap of the 6H4 epitope with the first segment of the 15B3 epitope (Fig. 2b).

Mapping of the 15B3 epitope onto the NMR structure of the C-terminal domain of mouse PrP (ref. 12) reveals close proximity of the peptide segments 2 and 3, but a much larger spatial separation of the segment 1 from either of the other two components (Fig. 3a). The peptide segment 1 occupies the N-terminal half of helix 1 plus the two residues preceding it, and it is recognized by 6H4 in PrP^C as well as by 15B3 in PrP^{Sc}. This finding would be compatible with either of the two following assumptions: (1) 15B3 recognizes segment 1 of its epitope only in concert with the segments 2 and 3; (2) the polypeptide segment of helix 1 is differently folded in PrP^C and PrP^{Sc}. Component 2 of the 15B3 epitope is in the loop connecting the second β-strand to the second helix, with two thirds of it in a disordered region in the three-dimensional structure, and component 3 is located at the C-terminal end of helix 3 (refs 12, 13). The peptide segments 2 and 3 are located in the proposed binding region for 'protein X' (ref. 14), which is characterized by significant alterations of the electrostatic surface potential among different mammalian species[15]: human PrP differs from bovine and mouse PrP in the replacement of the glutamine residues 168 and 219 by glutamic acid residues, as well as by conservative substitutions at positions 166 and 215. As bovine, mouse and human PrP^{Sc} are all precipitated by 15B3 (Fig. 1a–d), this antibody probably binds to the conserved residues in this region.

A single continuous 15B3 binding site could be formed either by aggregation of two or several PrP molecules[16], or by structural rearrangement of a single PrP molecule, or by a combination thereof. Figure 3b suggests a spatial arrangement of a PrP dimer that would bring all three segments of the 15B3 epitope into spatial proximity, with minimal conformational changes of the individual molecules. It is based on the observation of a structural similarity between PrP(121–231) and haemoglobins, which allows a superposition of the helices 1, 2 and 3 of PrP^C onto the helices 1, 6 and 7 of the haemoglobin β-subunit, with a root-mean-square distance for the polypeptide backbone of 2.4 Å. Superposition of two molecules

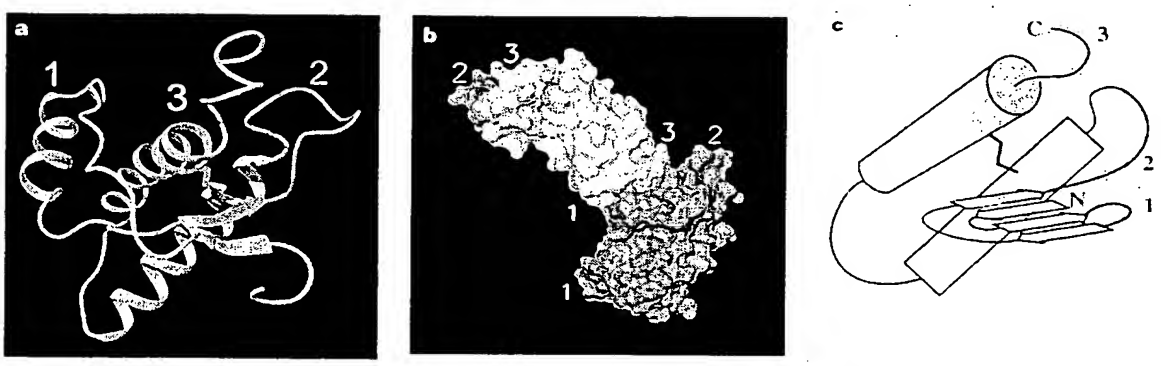

Figure 3 The epitope of the monoclonal antibody 15B3 in the three-dimensional prion protein structure. **a**, Mapping of the 15B3 epitope onto a ribbon drawing of the NMR structure of the C-terminal domain PrP(121-231) of mouse PrP^C (refs 12, 26). The three segments of the 15B3 epitope are coloured yellow (1), violet (2) and cyan (3) in the order in which they occur in the amino acid sequence (Fig. 2b). The residual parts of the molecule and the single disulphide bridge are grey. Regular secondary structures are indicated by ribbons for helices and arrows for β-strands. Drawings in **a** and **b** were prepared with MOLMOL[27]. **b**, Surface representation of two PrP(121-231) molecules after superposition onto two β-subunits of the crystal lattice of sickle cell haemoglobin[17] (Protein Data Bank entry 1HBS). The segments of the 15B3 epitope are numbered and coloured as in **a**. The superposition included the backbone atoms of residues 145-154, 179-189 and 201-217 of the helices 1, 2 and 3 of PrP(121-231) and of residues 5-14, 106-116 and 125-141 of the helices 1, 6 and 7 of haemoglobin S (r.m.s.d. = 2.4 Å). **c**, Hypothetical fold of the prion protein that would bring all three components of the 15B3 epitope into spatial proximity. The two cylinders represent the disulphide-linked helices 2 and 3 in the orientation of **a**. Helix 1 and the β-sheet have been replaced by four β-strands that form a greek key motif. The chain termini are labelled N and C, and the segments of the 15B3 epitope are numbered as in **a**.

of PrP(121–231) onto two adjacent β-chains in the crystal lattice of haemoglobin S[17] (PDB entry 1HBS) brings the peptide segment 1 of one PrP molecule near to the segments 2 and 3 of the other molecule (Fig. 3b). This superposition aligns residue 6 of the β-chains of sickle cell haemoglobin with Trp at position 145 of the mouse prion protein, which is located in the middle of the epitope segment 1 and is fully exposed to solvent. Mutation of Glu 6 to valine is responsible for the formation of haemoglobin aggregates in sickle cell anaemia.

Intramolecular structural rearrangement bringing all three segments of the 15B3 epitope into close spatial proximity might involve the first helix and lead to an extension of the existing β-sheet[13]. In Fig. 3c, the resulting β-structure is assumed to consist of four strands aligned to form a greek-key motif. Other PrP^{Sc} models that would also lead to close approach of the three segments of 15B3 epitope have been described[18].

The identification of an antibody that binds selectively to PrP^{Sc} from various species provides a new means to identify PrP^{Sc} directly without using proteinase K digestion as a criterion. It will be interesting to see whether 15B3 will be able to neutralize infectivity and thus be a potential therapeutic reagent. The low level of PrP^{Sc} in peripheral tissues has made it difficult to use it as a marker for prion diseases[19]. Affinity selection of PrP^{Sc} with 15B3 will allow enrichment of the abnormal isoform of PrP and thus lower the detection limit for PrP^{Sc}, so a prion test for living humans or animals is conceivable. The mapping and three-dimensional modelling of the 15B3 epitopes has provided a view of a prion-disease-specific epitope and may represent a starting point for the production of further diagnostic or therapeutic tools for TSEs. □

Methods

Materials. BSE material was from naturally occurring Swiss cases of BSE, CJD brain material from patients suffering from CJD type 1 (ref. 10), which had been diagnosed using histopathology and immunohistochemistry for PrP. For mouse scrapie material, CD-1 mice were experimentally infected with the RML strain[20].

Preparation of recombinant bovine PrP. The bovine PrP open reading frame was amplified by PCR from genomic DNA using the primers 5′-GGGAATTC-CATATGAAGAAGCGACCAAAACCTG and 5′-CGGGATCCTATTAACTTG-CCCCTCGTTGGTA. The resulting PCR product was cloned into pET11a (Novagen) using the *Nde*I and the *Bam*HI restriction sites. The resulting plasmid (pbPrP3) was transfected into *E. coli* BL21(DE3). Bacteria were grown to OD_{600} = 0.8 then induced with 1 mM IPTG and further grown at 30 °C for 3 h. rbPrP corresponding to the mature form of bovine PrP containing six octarepeats[21] was purified from inclusion bodies after solubilization in 8 M urea, 10 mM MOPS/NaOH, pH 7.0 (UM-buffer), on a CM sepharose column (Pharmacia; UM, 0–0.5 M NaCl gradient) and reverse-phase HPLC (Vydac C_4 column, 0.1% trifluoroacetic acid, 0–60% acetonitrile gradient; C.K. and B.O., unpublished results). Resulting fractions contained either oxidized (elution time, 29 min) or reduced PrP (elution time, 34 min). Usually, CM-Sepharose fractions were oxidized with 1 μM $CuSO_4$ for 1 h before purification by reverse-phase HPLC. Purified rbPRP was analysed by mass spectrometry, indicating a protein of the expected mass in which the N-terminal methionine was uncleaved.

Immunization of PrP-null mice. 100 μg recombinant bovine PrP in Freund's complete adjuvant was injected into PrP null mice (mixed background 129/Sv and C57BL/6J[22]) subcutaneously, and 21 and 42 d later with the same amount of antigen in Freund's incomplete adjuvant. Mice were boosted intraperitoneally (day 48) and intravenously (day 49) with recombinant PrP dissolved in PBS. At day 50, mice were decapitated and splenocytes fused to myeloma cells as described[23]. Supernatants of the resulting hybridoma cell lines were screened both by ELISA with recombinant bovine PrP as antigen and by ELIFA[24] using native, protease-digested brain homogenate of BSE-diseased cattle. Positive hybridoma cells were subcloned three times.

Characterization of antibodies. Supernatants of selected hybridomas were used to probe bovine PrP in brain homogenates or recombinant PrP on western blots. To determine the epitopes, antibodies were incubated with a gridded array of peptides comprising 104 polypeptides of 13 amino acids, shifted by two amino acids and covering the entire mature bovine PrP sequence containing six octapeptide repeats[21]. The peptides were covalently attached at their C termini to a cellulose support as individual spots (Jerini Biotools, Berlin). Bound antibody was detected with goat anti-mouse immunoglobulin coupled to horseradish peroxidase and chemiluminescence. Signals were recorded on Hyperfilm ECL (Amersham). For immunoprecipitation, 200 μl 1% brain homogenates (precleared by centrifugation at 13,000*g* for 15 min) were incubated for 2 h at room temperature with 200 μl 0.25 mg ml^{-1} antibody-containing serum-free medium; after incubation with an additional 50 μl protein A- or protein G-coupled agarose (for 15B3 and 6H4, respectively; Boehringer Mannheim) for 2 h at room temperature, agarose beads were centrifuged at 13,000*g* for 3 min and the pellet washed according to the manufacturer. Proteinase K (PK) digestions of immunoprecipitates were done with 20 μg ml^{-1} PK (Sigma) for 30 min at 37 °C. Pellets were then boiled in SDS-sample buffer for analysis on western blots. Immunoprecipitated PrP was detected with polyclonal antibodies raised against bovine recombinant PrP in rabbits (R 26) followed by incubation with a goat anti-rabbit immunoglobulin coupled to peroxidase or a goat anti-rabbit IgG coupled to alkaline phosphatase. Bound enzymatic activity was visualized with chemiluminescent substrates (ECL, Amersham, or CSPD, Tropix, respectively).

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NMR structure of the mouse prion protein domain PrP(121–231)

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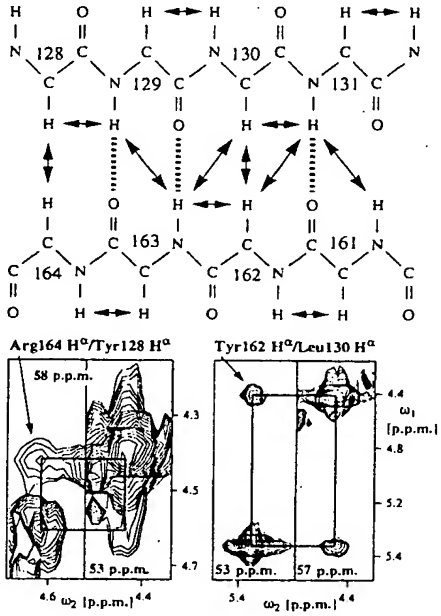
THE 'protein only' hypothesis[1] states that a modified form of normal prion protein triggers infectious neurodegenerative diseases, such as bovine spongiform encephalopathy (BSE), or Creutzfeldt–Jakob disease (CJD) in humans[2–4]. Prion proteins are thought to exist in two different conformations[5]: the 'benign' PrP^C form, and the infectious 'scrapie form', PrP^{Sc}. Knowledge of the three-dimensional structure of PrP^C is essential for understanding the transition to PrP^{Sc}. The nuclear magnetic resonance (NMR) structure of the autonomously folding PrP domain comprising residues 121–231 (ref. 6) contains a two-stranded antiparallel β-sheet and three α-helices. This domain contains most of the point-mutation sites that have been linked, in human PrP, to the occurrence of familial prion diseases[7]. The NMR structure shows that these mutations occur within, or directly adjacent to, regular secondary structures. The presence of a β-sheet in PrP(121–231) is in contrast with model predictions of an all-helical structure of PrP^C (ref. 8), and may be important for the initiation of the transition from PrP^C to PrP^{Sc}.

The NMR structure of PrP(121–231) (Fig. 1*a* and Table 1) contains three α-helices and a two-stranded antiparallel β-sheet. The approximate lengths of the helices are from residues 144 to 154, 179 to 193, and 200 to 217, and the lengths of the β-strands are from residues 128 to 131, and 161 to 164. The first turn of the second helix and the last turn of the third helix are linked by the single disulphide bond in the protein. The twisted V-shaped arrangement of these two longest helices forms the scaffold onto which the short β-sheet and the first helix are anchored. At the present stage of refinement, all regular secondary-structure elements and the connecting loops are well defined (see Table 1 and Fig. 1*d*, *e*), with the sole exception of residues 167 to 176. The polypeptide fold is stabilized by hydrophobic interactions in a core that contains side chains of the second helix (residues 179, 180 and 184), the third helix (residues 203, 206, 209, 210, 213 and 214), the β-sheet (Val 161), the first, mostly hydrophilic, helix (Tyr 150), and three loop regions (residues 134, 137, 139, 141, 157, 158 and 198). With the exceptions of Ile 139, Ile 184 and Val 203, the residues of the hydrophobic core are invariant in the known mammalian prion protein sequences[9] (Fig. 3*a*). Hydrophobic surface patches in PrP(121–231) are located near the β-sheet and the loop preceding the first helix. The surface of PrP(121–231) is otherwise characterized by a markedly uneven distribution of positively and negatively charged residues (Fig. 1*b*, *c*).



Mature mouse PrP^C is a glycosylated 208-residue protein (codons 23–231, with deletion of codon 55 (ref. 9)) that is attached to the cell surface by means of a glycosyl phosphatidyl inositol anchor at its carboxy-terminal Ser 231 (ref. 10). It seems to be necessary for normal synaptic function[11] (but see ref. 12), long-term survival of Purkinje neurons[13], and the regulation of circadian activity rhythms and sleep[14]. The segment of residues 109–218 was predicted to form a four-helix-bundle with helices at residues 109–122, 129–141, 178–191 and 202–218 (ref. 8), whereas prediction algorithms did not yield conclusive results for the segment 23–108, which contains the prion-characteristic octapeptide repeats. Attempts to express PrP(108–231) in the periplasm of *Escherichia coli* resulted in proteolytic cleavage after residues 112, 118 and 120, whereas PrP(121–231) is stable against degradation in *E. coli*, folds cooperatively and reversibly at pH 7 ($\Delta G_{Fold.} = -22$ kJ mol^{-1}), and is soluble at 1 mM concentration in distilled water between pH 4.0 and pH 8.5 (ref. 6). This segment also contains six of nine point-mutation sites in mature PrP that have been associated with familial prion diseases[7] (Fig. 3*a*), as well as both glycosylation sites of PrP and its single disulphide bond[10]. We therefore chose to use PrP(121–231) for the present NMR structure determination. This choice was also supported by the demonstration that the segment 81–231 of mouse PrP is sufficient for propagation of the prion disease *in vivo*[15], indicating that the C-terminal part of PrP is of special functional importance.

In the context of the structure predictions for PrP^C (ref. 8), the β-sheet in the NMR structure of PrP(121–231) is an unexpected feature. Evidence for the identification of the β-sheet is shown in

TABLE 1 Parameters characterising the NMR structure determination of PrP(121–231)

Extent of assignments (backbone and side chain ^{1}H, $^{13}C^\alpha$, backbone ^{15}N)	93%
Number of distance constraints	1,368
Number of dihedral angle constraints	227
Distance constraint violations >0.1 Å (per conformer)	1.5 ± 1.3
Dihedral angle constraint violations > 2.5° (per conformer)	0.15 ± 0.36
Intra-protein AMBER energy (kcal mol^{-1})	−5,041 ± 97
R.m.s.d. to the mean for N, C^α and C′ of residues 125–166 and 177–219	1.4 Å
R.m.s.d. to the mean for all heavy atoms of residues 125–166 and 177–219	2.0 Å

The NMR structure of PrP(121–231) was calculated with the program DIANA[21]. Starting from 100 randomized structures, the 20 conformers with the lowest DIANA target function values were energy minimized in a water shell of 6 Å minimal thickness, using the program OPAL (P. Luginbühl, P. Güntert, M. Billeter and K. Wüthrich, submitted) with the AMBER force field[22].

FIG. 2 Identification of the antiparallel β-sheet in PrP(121–231). Top, β-sheet with arrows showing the NOEs from which this structure was identified before the structure calculation[16]. Broken lines indicate β-sheet hydrogen bonds for which slowed amide–proton exchange was observed. Bottom, spectral regions from a 3D ^{13}C-correlated [^1H, ^1H]-NOESY spectrum recorded in D_2O solution; squares connect the cross peaks and diagonal positions for the $d_{\alpha\alpha}(128, 164)$ and $d_{\alpha\alpha}(130, 162)$ connectivities.

Fig. 2, including the raw data on the two $d_{\alpha\alpha}$ interstrand nuclear Overhauser enhancement (NOE) connectivities, which are the most direct NMR identifiers of antiparallel β-structure[16]. Considering the proposed increase of the β-sheet content in PrP shown in transition from PrP^C to PrP^{Sc} (refs 5,17), it is tempting to speculate that the short β-sheet shown in Fig. 2 might be a 'nucleation site' for a conformational transition that could include the loops connecting the β-sheet to the first helix, which is predominantly hydrophilic and does not show amphipathic character. A systematic search of the Brookhaven data bank[18] with the program DALI[19] did not lead to the identification of other

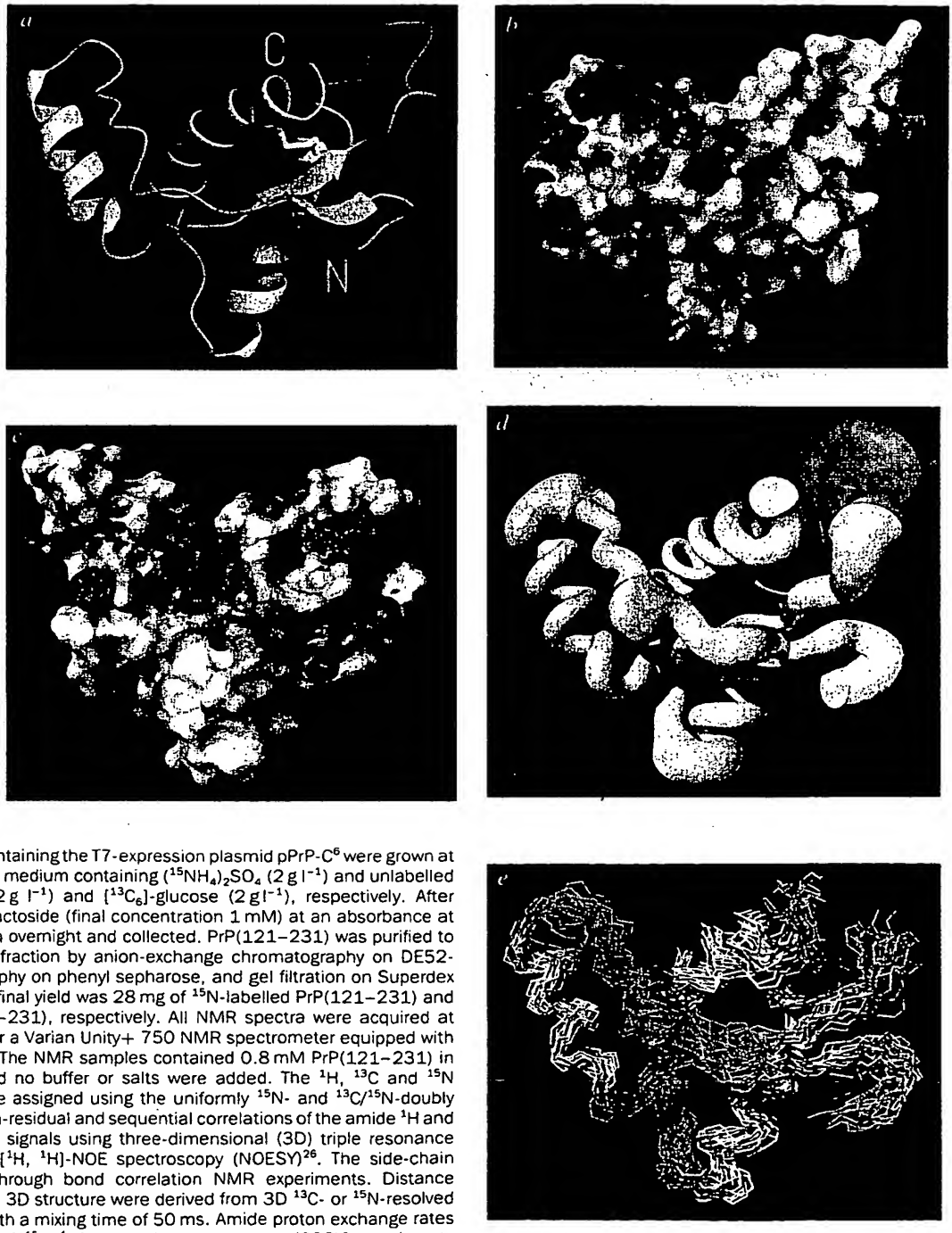
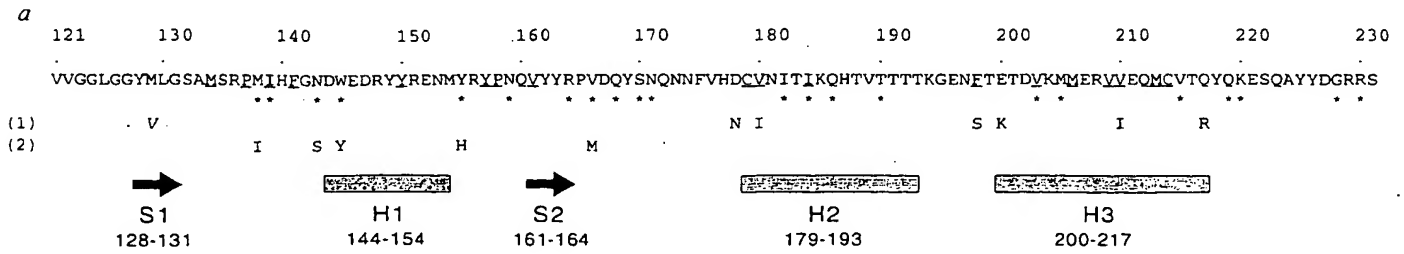


FIG. 1 Globular fold and surface properties of PrP(121–231). *a*, Ribbon diagram of the structure of the mouse prion protein domain PrP(121–231), indicating the positions of the three helices (yellow) and the antiparallel two-stranded β-sheet (cyan). The connecting loops are displayed in green if their structure is well defined, and in magenta otherwise. The disulphide bond between Cys 179 and Cys 214 is shown in white. The N-terminal segment of residues 121–124 and the C-terminal segment 220–231 are disordered and not displayed. *b*, *c*, Surface of the structure of PrP(121–231). Colours indicate the electrostatic potential[23], with blue for positive charges, red for negative charges. *b*, Same orientation as *a*. *c*, View after 180° rotation about a vertical axis. *d*, *e*, Representations of the precision of the structure determination. *d*, Display of the backbone of PrP(121–231) as a cylindrical rod of variable radius, which represents the global displacements among the 20 conformers used to represent the NMR structure (Table 1)[24]. Same orientation as *a*. Yellow represents residues 125–166 and 177–219, and the disordered loop of residues 167–176 is shown in magenta. *e*, Superposition of the 20 conformers; colours as in *a*.

METHODS. For the production of uniformly ^{15}N-labelled and $^{15}N/^{13}C$-doubly labelled PrP(121–231), cells of *E. coli* BL21(DE3)[25] containing the T7-expression plasmid pPrP-C[6] were grown at room temperature in 10 l of minimal medium containing $(^{15}NH_4)_2SO_4$ (2 g l^{-1}) and unlabelled glucose (5 g l^{-1}) or $(^{15}NH_4)_2SO_4$ (2 g l^{-1}) and [$^{13}C_6$]-glucose (2 g l^{-1}), respectively. After induction with isopropyl-β-D-thiogalactoside (final concentration 1 mM) at an absorbance at 550 nm of 0.7, the cells were grown overnight and collected. PrP(121–231) was purified to homogeneity from the periplasmic fraction by anion-exchange chromatography on DE52-cellulose, hydrophobic chromatography on phenyl sepharose, and gel filtration on Superdex 200, as described elsewhere[6]. The final yield was 28 mg of ^{15}N-labelled PrP(121–231) and 16 mg of $^{13}C/^{15}N$-labelled PrP(121–231), respectively. All NMR spectra were acquired at 20 °C either on a Bruker AMX 600 or a Varian Unity+ 750 NMR spectrometer equipped with triple-resonance *z*-gradient probes. The NMR samples contained 0.8 mM PrP(121–231) in 90% H_2O/10% D_2O at pH 4.5, and no buffer or salts were added. The ^1H, ^{13}C and ^{15}N resonances with the backbone were assigned using the uniformly ^{15}N- and $^{13}C/^{15}N$-doubly labelled proteins by establishing intra-residual and sequential correlations of the amide ^1H and ^{15}N resonances with C^α, C^β and H^α signals using three-dimensional (3D) triple resonance experiments and 3D ^{15}N-resolved [^1H, ^1H]-NOE spectroscopy (NOESY)[26]. The side-chain signals were assigned from 3D through bond correlation NMR experiments. Distance constraints for the calculation of the 3D structure were derived from 3D ^{13}C- or ^{15}N-resolved [^1H, ^1H]-NOESY spectra recorded with a mixing time of 50 ms. Amide proton exchange rates were measured by recording a series of [^{15}N, ^1H]-correlation spectroscopy (COSY) experiments immediately after dissolving lyophilized PrP(121–231) in D_2O. The program MOLMOL[24] was used to generate the figure.



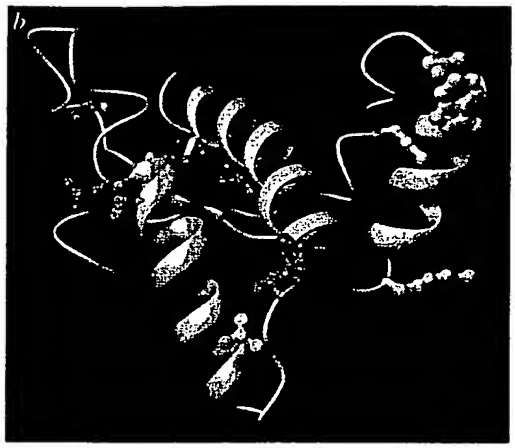


FIG. 3 Location in the 3D structure of PrP(121–231) of residues involved in sequence variations among mammalian prion proteins, and of residues that have been associated with the species barrier of prion disease transmission and with inherited prion diseases. *a*, Sequence and regular secondary structure of mouse PrP(121–231). Residues contributing to the hydrophobic core of the domain are underlined; variable residues among mammalian prion proteins[9] are marked with asterisks. Line (1), mutations in human PrP that have been associated with inherited prion diseases[7] (a stop codon at residue 145, which has been reported in addition to these point mutations[7], is not considered here, nor is the Met232Arg mutation[7], which is not contained in mature PrP^C). All of these residues are identical in wild-type human and mouse PrP. The polymorphism at codon 129 in human PrP, where homozygosity appears to increase susceptibility to sporadic CJD[7], is marked by italics. Line (2), residues in PrP(121–231) for which experimental evidence has been presented that they contribute to the species barrier of prion disease transmission between mice and humans[20]. *b*, Locations of selected residues in the three-dimensional structure of PrP(121–231). The backbone is shown in grey and the orientation of the molecule is as in Fig. 1c. The side chains of amino-acid residues with mutations that have been associated with inherited prion diseases[7] are highlighted in red (line (1) in *a*). The solvent-accessible glycosylation sites at Asn 181 and Asn 197 are shown in green, and the disulphide bond is shown in yellow. Five residues that may be involved in the species barrier (line (2) in *a*) are shown in blue. Figure generated using the program MOLMOL[24].

proteins with folds similar to PrP(121–231), and the relative orientation of the three helices in PrP(121–231) is clearly different from the proposed four-helix-bundle model[8].

Mapping onto the three-dimensional structure of PrP(121–231) of sequence variability in mammalian prion proteins[9], of residues important for the species barrier of prion disease transmission[20] and for predisposition to familial prion diseases[7], and of biochemical properties of the prion protein[6,10] (Fig. 3*a*, *b*) shows the following. (1) Invariant residues in mammalian prion protein sequences are not clustered within the regions of regular secondary structure, but form an important part of the hydrophobic core. (2) The two glycosylation sites at Asn 181 and Asn 197 and the solvent-exposed, single Trp 145 are located on the negatively charged surface of the protein. (3) All six residues of PrP(121–231) for which mutation is believed to be associated with inherited prion diseases or predisposition to prion diseases are located in regular secondary structure elements or immediately adjacent to them, but none of these residues is located in the relatively isolated first helix. Three of these residues are part of the hydrophobic core, and three are located on the surface. They may therefore either destabilize the three-dimensional protein structure, or influence its ligand-binding properties. (4) As is generally observed for functionally related proteins from different species, residues in PrP(121–231) that are variant in mammalian prion protein sequences[9] are solvent accessible. (5) The disulphide bond 179–214 is highly shielded from solvent contact in the core of the protein.

It has been proposed that the species barrier of prion disease transmission between mice and humans is caused by an altered PrP^{Sc} binding site in PrP^C, which involves residues from the segment 96–167 (ref. 20). There are eight sequence differences between mouse and human PrP within this segment, of which five are contained in PrP(121–231). Four of these differences are located within or adjacent to the first helix, which might thus be part of a single binding site for PrP^{Sc} (Fig. 3*a*, *b*). The dipolar character of PrP(121–231) (Fig. 1*b*, *c*) might stabilize an orientation of PrP^C with its positively charged surface, which also includes hydrophobic surface patches, towards the cell membrane. Both glycosylation sites, as well as the aforementioned species barrier-related potential binding site for PrP^{Sc}, would then be located on the opposite, negatively charged surface. Further to these initial observations on possible structure–function correlations, we believe that the NMR structure of PrP(121–231) will provide a basis for more rational design of future *in vitro* and *in vivo* experiments on prion proteins and prion diseases. □

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